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Note

A protocol for direct sequencing of multiple gene specific PCR products from *Discula umbrinella*, a fungal endophyte, utilizing bufferless precast electrophoresis

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Abstract

A protocol for direct sequencing of polymerase chain reaction (PCR) products from mycelia of *Discula umbrinella*, a fungal endophyte, using bufferless electrophoresis is described. This improved method allows researchers to conduct high-capacity screening of multiple gene regions for fungal endophytes applicable to microbial ecology and population genetic studies.

Keywords: Sequence; PCR; *Discula umbrinella*; Endophyte

Direct sequencing of polymerase chain reaction (PCR) products and separation of products by bufferless electrophoresis (without buffer reservoirs) can be important tools for large-scale microbial ecology studies with endophytic fungi. These fungal species reside within living plant tissue, while frequently providing plants with protection against insects by releasing anti-herbivore chemicals and/or reducing the survival of insect larvae by pre-colonizing plant tissue (Clay, 1992; Petrini, 1995). Traditionally, researchers have isolated fungal endophytes on agar media from plant material and identified fungal isolates based on morphological features. In many cases, endophytes grow slowly and are difficult to isolate from complex microbial communities residing on native substrates. This fact has encouraged researchers to look for effective methods to track the identity and population changes of fungal endophytes in nature. Several molecular methods have proven to be effective for fungal identification, studying fungal growth and fungal–plant interactions and detection of fungal endophytes in natural plant substrates (Adair et al., 2002; Mazzaglia et al., 2001; Hammerli et al., 1992; Heuser and Zimmer, 2002; Viaud et al., 2000; Ward and Adams, 1998). For example, Mazzaglia et al. (2001) developed a PCR assay for selective detection of *Biscogniauxia mediterranea*, an endophyte of *Quercus* species. This assay is designed to distinguish

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the change from endophytic stage to pathogenic stage of this fungus, as the plant host becomes water stressed. Hammerli et al. (1992) determined fungal-host specificity of *Discula umbrinella* isolates collected from various tree species using randomly amplified polymerase chain reaction products (RAPDs).

For this study with *D. umbrinella* in oak, a direct sequencing protocol was developed based on modifications of DNA isolation and bufferless electrophoresis for separation of PCR products. Improvements in DNA extraction from *D. umbrinella* were achieved by grinding fresh mycelia with a chip of dry ice in a prechilled, sterile mortar and pestle. This process effectively released the DNA from cell debris for isolation and purification. Further DNA purification steps followed a protocol developed by Li and Chory (1998) with some modifications. Protocols for amplification of PCR products followed those developed by Carbone and Kohn (1999). Bufferless electrophoresis with precast agarose E-Gels was used to separate PCR products from DNA regions of chitin synthase (CHS-1), translation elongation factor 1-alpha (EF-1α) and the intergenic spacer region (IGS) of ribosomal DNA isolated from *D. umbrinella*. PCR products were eluted from agarose and purified prior to DNA sequencing. Bufferless precast agarose electrophoresis was included in the protocol in order to screen for any artifacts and to demonstrate that products from gene specific PCR primers produced only single DNA bands.

D. umbrinella isolates (WO 122 and RO 226) were cultured from leaves of two species of oaks, *Quercus alba* and *Quercus rubra*, located at Patuxent Wildlife Research Center, Laurel, Maryland, USA. Isolation of fungal cultures, maintenance of cultures, and techniques for leaf collection prior to fungal isolation were previously described (Cohen, 1999). Cultures were grown for 7 days at 24 °C on a rotary shaker (100 rpm) in 50 ml of Czapek-Dox liquid media (glucose, 30 g; yeast extract, 5 g; $NaNO_3$, 3 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_20$, 0.5 g; $FeSO_4 \cdot 7H20$, 0.01 g and 1000 ml deionized water). Genomic DNA was extracted from culture samples according to the isolation procedure listed in Table 1. Isolation of DNA from strains of *D. umbrinella* (Berk. et Broome) Sutton proved to be difficult using a grinding procedure with sand or glass beads in a mortar chilled by liquid nitrogen and followed by a CTAB extraction protocol (Hammerli et al., 1992). Using a tissue grinder, a mini bead beater (3 mm glass beads), electric stirrer with teflon pestle (3 mm glass beads) and mortar and pestle with glass beads (3 mm) were also not effective for disrupting the cell walls of the fungus. The presence of sand or glass beads did not adequately break up hyphal walls, as determined by microscopic examination of the extraction slurry. In addition, the mass of debris produced by grinding hindered the release and extraction of DNA. Substituting a chip of dry ice as the grinding medium in a precooled −80 °C mortar significantly improved release of cytoplasmic contents from hyphae (Table 2). Extraction of DNA (quality and quantity) from mycelial extract was further enhanced by incubating at 65 °C in a SDS-buffer for 10 min with occasional inverted mixing. Typically, DNA extracts isolated from 250 mg of freshly harvested mycelia contained 2.5–5.0 μg of genomic DNA. Isolation of genomic DNA was more easily accomplished from fresh mycelia than freeze-dried mycelia.

Genomic DNA isolated using the described protocol without an additional purification step using the Gene Clean kit (Bio 101, Vista, CA) was suitable for amplification and sequencing of the translation elongation factor 1-alpha (EF-1α) (GenBank: AF229164, AF229165) and chitin synthase (CHS-1) (GenBank: AF231712, AF231713). Further purification of genomic DNA using Gene Clean was required prior to amplification of the intergenic spacer region (IGS) of rDNA (GenBank: AF235163, AF235164) to eliminate the potential for single-primer PCR product formation.

Quantification of DNA in samples was determined by UV absorbance using a Beckman DU-7 spectrophotometer. DNA yields were calculated on the basis of UV absorbance×dilution and purity of the samples was estimated by the ratio of UV absorbance at A_{260}/A_{280}. DNA samples were diluted to 5 ng/μl prior to amplification and amplification reactions and PCR cycle conditions were used as described in Table 1. The primers used in the amplification reactions were: (1) translation elongation factor EF1-α, 5′-CATCGAGAAGTTCGAGAAGG (EF 1-728 F, forward primer) and 5′-TACTTGAAGGAACCCTTACC (EF 1-986 R, reverse primer); (2) chitin synthase 1 primers

Table 1
D. umbrinella protocol for DNA isolation and amplification

Procedure steps	
Solutions	
Lysis buffer	0.1 M Tris, 0.01 M EDTA, 0.5 M NaCl at pH 8.0 0.01 M 2-mercaptoethanol and 20% SDS
5 M KOAc	
3 M NaOAc	
Isopropanol	
70% Ethanol	
0.01 M Tris, 0.001 M EDTA at pH 8.0	
DNA isolation[a]	
(1) Cultures were harvested onto Miracloth by vacuum filtration under laminar flow hood, rinsed three times with sterile water, and 250 mg sample taken, frozen at −20 °C before DNA extraction. Samples were then removed from −20 °C, placed in a prechilled (−80 °C) mortar and pestle with a pellet of dry ice and ground to a fine powder. The powdered samples were placed in sterile 15 ml centrifuge tubes and returned to −80 °C prior to extraction.	
(2) Lysis buffer (750 μl) containing Tris, EDTA, NaCl and mercaptoethanol was added to the 250 mg sample removed from −80 °C freezer and vortexed for 30 s; 50 μl of SDS was added to the sample, mixed gently, and heated for 10 min at 65 °C with periodic mixing.	
(3) Sample was transferred to microcentrifuge tubes and 270 μl of potassium acetate were added, tubes were shaken then left on ice for 20 min. Samples were centrifuged at 14,000×*g* for 15 min.	
(4) Supernates were transferred to new tubes, placed on ice for 5 min and isopropanol precooled to −20 °C was added to samples, mixed by inversion. Sodium acetate was then added to the samples, mixed by inversion and left on ice for 5 min. Prechilled isopropanol was added to samples, mixed and left on ice for 1 h.	
(5) Samples were centrifuged for 5 min at 14,000×*g*, the supernate was removed and 1 ml of 70% ethanol was added. Samples were again centrifuged for 5 min at 14,000×*g* and supernate was removed by aspiration.	
(6) Samples were allowed to air dry for 30 min and the pellets were resuspended in 0.01 M Tris, 0.001 M EDTA, pH 8.0 at 37 °C for 1 h and then heated to 65 °C for 1 min. Samples were then stored in a refrigerator at −4 °C. A gene clean kit (BIO 101) was used to further purify genomic DNA samples prior to amplification of the intergenic spacer region of ribosomal DNA.	
Solutions	
0.5 μM primers[b]	
200 μM dNTPs	
PCR buffer II	
2 mM $MgCl_2$	
0.5 U of AmpliTaq Gold DNA polymerase	
25 ng genomic DNA	
DNA amplification[b]	
(1) Each amplification reaction contained the above components in a 25 μl volume.	
(2) Amplifications were carried out in a 2400 thermocycler (Perkin-Elmer).	
(3) Initial denaturation at 95 °C for 10 min followed by 35 cycles of 95, 55, and 72 °C for 15, 20 and 60 s with a 5-min extension at 72 °C on the final cycle.	

[a] DNA isolation procedure modified from Li and Chory (1998).
[b] Primers and PCR cycle conditions from Carbone and Kohn (1999).

consisted of 5′-TGGGGCAAGGATGCTTGGAAGAAG (CHS-79 F, forward primer) and 5′-TGGAAGAACCATCTGTGAGAGTTG (CHS-354 R, reverse primer); and (3) primers used to amplify the intergenic spacer region of rDNA were 5′-AGTCTGTGGATTAGTGGCCG (IGS-12 AF, forward primer) and 5′-GAGACAAGCATATGACTAC (NS1R, reverse primer).

Amplified samples were prepared for gel electrophoresis by mixing 5 μl of the sample with loading buffer and water. Either 250 ng of a 1-kb ladder standard (Gibco-BRL) or fungal DNA sample was loaded into each sample well of a 12-well, 1.2% agarose E-GEL containing ethidium bromide stain (0.1–0.3 μg/ml) (Invitrogen, Carlsbad, CA). The gel was run for 30 min under constant current 40–50 mA and results photographed using a video camera and a UV transilluminator.

For sequence analysis, duplicate amplified reactions (90 μl) were pooled, loaded on a 1.2% agarose E-gel

Table 2
Comparison of methods of macerating mycelia of *D. umbrinella* into hyphal fragments

Method[a]	Microscopic observation[b]	Presence of DNA[c]	DNA yield[d]
Mini bead beater	+	–	0
Glass homogenizer	+	–	0
Electric stirrer/ teflon pestle	+	–	0
Dry ice chip/ mortar/pestle	+++	+	17±4.7

[a] Mini Bead Beater (Biospec, Bartlesville, OK) and Electric Stirrer/Teflon Pestle methods contained glass beads (3 mm in size).
[b] Degree of mycelia fragmentation by microscopic visual observation at 100× magnification: 0–10% of mycelia fragmented (+), 80% of the mycelia fragmented (+++).
[c] Presence (+) or absence (−) of genomic DNA detected on 1% bufferless precast agarose gel prestained with ethidium bromide (0.1–0.3 μg/ml).
[d] Mean (±S.D.) DNA yield (μg DNA/g dry weight) of four replicate samples measured in sample preparations with UV absorbance at A_{260}.

and then resolved by electrophoresis for 30 min at 40–50 mA. The gel cassette was opened, DNA bands were excised from the gel for immediate extraction or stored at −20 °C for future extraction. DNA was purified from gel slices using the Gene Clean Kit (BIO 101). DNA concentration was determined by the UV absorbance using a Beckman DU-7 spectrophotometer. The purified PCR products were sequenced with forward and reverse primers on an ABI 7700 sequencer. Sequence chromatograms were edited with Chromas software (http://www.technelysium.com.au/chromas.htmlhttp://www.technelysium.com.au/chromas.html) and aligned pairwise with GeneDoc software (http://www.psc.edu/biomed/genedoc). All sequences were analyzed for homology by using the nucleotide–nucleotide "BLAST" search feature located on the NCBI web site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Percent of homology to published sequences ranged from 87% to 95%. Expected values of homology were very significant for regions CHS-1 and IGS and less significant for the EF-1α region (Table 3).

In summary, improvements in a number of steps for the direct sequencing protocol facilitated isolation and purification of genomic DNA, and separation of PCR products. These improvements included grinding freshly harvested hyphae with dry ice under cold conditions, modification of the existing DNA isolation techniques, separation of PCR products by bufferless electrophoresis, and purification of PCR products with GeneClean. This protocol, designed for direct sequencing of multiple gene regions using bufferless electrophoresis with precast agarose E-Gels, is easily adaptable to large-scale microbial ecology studies with endophytic fungi and may be applicable for future high throughput procedures.

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Table 3
Direct sequencing of PCR products from *D. umbrinella* using bufferless precast electrophoresis

Isolate[a]	GenBank	PCR product (ng/μl)[b]	A_{260}/A_{280}	Size (bp)	Homology (%)	Expect value ("BLASTN")
WO 122						
EF-1α	AF229164	22.5	3	341	95	0.20
CHS-1	AF231712	100	1.866	250	87	1e−48
IGS	AF235163	25	1.75	363	94	2e−11
RO 266						
EF-1α	AF229165	127.5	2.117	341	95	0.20
CHS-1	AF231713	182.5	1.888	250	87	1e−48
IGS	AF235164	57.5	2.66	363	94	2e−11

[a] WO 122: fungal isolate cultured from a leaf of *Q. alba* (white oak); RO226: fungal isolate cultured from a leaf of *Q. rubra* (red oak); gene regions: translation elongation factor (EF-1α), chitin synthase (CHS1), intergenic spacer region (IGS).
[b] Concentration of PCR product eluted from gel slice in 20 μl of sterilized water.

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